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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 5 :		(11) International Publication Number:	WO 92/15327
A61K 39/00	A1	(43) International Publication Date:	17 September 1992 (17.09.92)

(21) International Application Number: PCT/US92/01784

(22) International Filing Date: 6 March 1992 (06.03.92)

(30) Priority data:
666,287 8 March 1991 (08.03.91) US

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: RECOMBINANT DOUBLE CHAIN IMMUNOTOXINS

(57) Abstract

Recombinant immunotoxins are provided having a double chain antigen binding component, typically an immunoglobulin or portion thereof, one chain of which is in peptide linkage to a bacterial toxin. Also provided are therapeutic compositions and methods employing such immunotoxins.

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RECOMBINANT DOUBLE CHAIN IMMUNOTOXINS

Field of the Invention

The present invention relates generally to recombinant double chain immunotoxins and methods for their production and use.

BACKGROUND OF THE INVENTION

An immunotoxin is a chimeric compound consisting of a toxin linked to an antibody having a single desired specificity, notably characteristic antigenic determinants expressed on the surface of certain cells, including microorganisms, neoplastic cells, virally infected cells, and subsets of normal cells. Such antibodies thus act as targeting agents for the toxin, allowing it to act cell-specifically (for reviews, see I. Pastan et al., Cell 47:641-648 (1986); and E. Vitetta et al., Science 238:1098-1104 (1987), both incorporated herein by reference).

Toxins are products of nature usually produced by bacteria and plants (S. Olsnes and A. Pihl, in Molecular Action of Toxins and Viruses, P. Cohen and S. van Heyningen, eds. (Elsevier, New York, 1982), p. 503, incorporated herein by reference). Among the best studied and most widely employed toxins are the plant-derived toxic lectins, notably ricin, but also including abrin and modeccin. Toxic lectins consist of two polypeptide chains, A and B, linked by disulfide bridge(s), of which the A chain is cytotoxic via its ability to block ribosomal protein synthesis in eukaryotic cells. The B chain recognizes polysaccharide units at the cell surface and creates a high affinity binding to such units. The complex of toxin and cell surface glycoprotein to which it is bound is endocytosed, the disulfide bond between the A and B chains is reduced, and the A chain translocates across an endocytic membrane to gain access to the cytosol.

A wide variety of other toxins, including bacterial toxins (e.g., diphtheria toxin), have also been studied. Some of these, such as Pseudomonas Exotoxin A, consist of a single polypeptide chain having both binding and cytotoxic activities.

Most immunotoxins have been made with one of a limited class of toxins that act to directly inhibit protein synthesis by inactivating ribosomes (e.g., ricin) or elongation-factor-2 (e.g., diphtheria toxin, Pseudomonas exotoxin A). These toxins must be internalized into the cytoplasm before damaging a cell. Moreover, they have cell binding domains that must be removed to prevent unacceptable toxicity toward normal cells.

Immunotoxins prepared with ricin or its A chain have been most widely used to date, and the ricin A chain is usually coupled to a cell-reactive antibody by chemically cross-linking ricin or its A chain to the antibody, e.g., through thioether linkages. At present, immunotoxins made with intact ricin appear to be prohibitively toxic for systemic administration (E. Vitetta et al. (1987)). In contrast, immunotoxins containing only the ricin A chain have exquisite specificity, but show unpredictable cytotoxicity because of the absence of B chain-mediated potentiation of A chain translocation (Reviewed in Immunol. Rev. 62 (1982). See also, R. Youle and D. Neville, Jr., J. Biol. Chem. 257:1598 (1982); and D. McIntosh et al., Fed. Eur. Biochem. Soc. 164:17 (1983); all incorporated herein by reference).

Immunotoxins made with antibodies directed toward tumor-associated antigens or lymphoid markers can selectively eliminate cancer cells or subsets of lymphoid cells in vitro and in vivo. Such immunotoxins hold considerable promise for the treatment of cancer and autoimmune conditions in human patients. However, in actual clinical trials with cancer patients, the benefits have so far been very modest (E. Vitetta et al. (1987)). This lack of dramatic clinical success has been attributed to the inaccessibility of the tumor tissue to the larg immunotoxin molecules, and to insufficient toxicity of the immunotoxin toward cancer cells relative to normal cells. Moreover, immunotoxins have commonly been made by

chemically attaching a toxin to an intact antibody containing th constant region of the antibody, which is not nec ssary for immunotoxin action, but which reduces its access to target cells outside the circulation, and increases its immunogenicity. The product is heterogenous and unstable, and the yields are often poor.

Recently, a single chain immunotoxin was prepared by recombinant means, namely a single chain anti-Tac(Fv)-PE40 having the two variable regions joined by a linker and the toxin polypeptide in peptide linkage with one of the two variable segments (V. Chaudhary et al., Nature 339:394 (1989), incorporated herein by reference).

Immunotoxins exhibiting improved characteristics are still actively sought for a number of reasons. Immunotoxins coupled by chemical conjugation are heterogenous and unstable, and thus may be dangerous to the patient. They are also relatively expensive to produce. Small, single-chain immunotoxins have a relatively short half-life, which typically impairs efficiency. Moreover, the unnatural links between variable domains in the single chain recombinant immunotoxin often leads to a reduced affinity for the antigenic determinant recognized by the antigen binding site. There exists a need, therefore, for improved immunotoxin compositions that avoid the problems associated with chemically coupling toxin moieties to immunoglobulins, while also providing the advantages of higher affinities for the target cell and a longer half-life. One would thus be able to maintain efficacy at lower doses, reducing side effects of treatment. The present invention fulfills these and other needs.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic diagram of anti-Tac(Fab)-PLC genetic construct (SEQ. ID NOS:1 and 2). Not drawn to scale. The mature protein coding sequences are shaded. The DNA linker between PLC and the anti-Tac heavy chain is shown with ApaI and BstEII sites underlined. Relevant res_riction sites are indicated. STII, E. coli heat-stable enterotoxin II; S.D.,

Shine and Dalgarno sequence; E, EcoRI; A, ApaI; B, BstEII; X, XbaI; M, MluI.

Fig. 2. Purification of anti-Tac(Fab)-PLC. Samples were analyzed on a 10% SDS polyacrylamide gel and stained with Coomassie blue. Lane 1, osmotic shock fluid, non-reduced; lane 2, purified anti-Tac(Fab)-PLC, non-reduced; lane 3, protein markers with molecular weights 94, 67, 43, 30, 20, 14 kD, reduced; lane 4, purified anti-Tac(Fab)-PLC, reduced. The arrow indicates the light chain band of 25 kD.

Fig. 3. Competitive binding of anti-Tac and anti-Tac(Fab)-PLC to HuT-102 cells. ■, anti-Tac competitor; ●, anti-Tac(Fab)-PLC competitor.

Fig. 4. Cytotoxicity of PLC (○), anti-Tac(Fab)-PLC (●), and anti-Tac(Fab)-PLC plus 10 ug/ml anti-Tac (■) on HuT-102 cells. Results are expressed as percent of the maximum ^{3}H-leucine uptake.

Fig. 5. Schematic diagram of the anti-Tac(Fab)-PE40 immunotoxin. V_H, variable domain of anti-Tac heavy chain; V_L, variable domain of anti-Tac light chain; C_H1, first constant domain of anti-Tac heavy chain; C_L, constant domain of anti-Tac light chain; S-S, disulfide bond; PE40, Pseudomonas exotoxin-40.

Fig. 6. Schematic diagram of DNA construct for anti-Tac (Fab)-PE40 (SEQ. ID NOS:3 and 4). Protein designations as in Fig. 5. The linker sequence inserted between V_LC_L and PE40 is shown, with the NcoI site underlined. STII, E. coli heat stable enterotoxin II; S.D., Shine and Dalgarno sequence; E, EcoRI; B, BstEII; X, XbaI; M, MluI; N, NcoI.

Fig. 7. Cytotoxicity of anti-Tac(Fab)-PE40 alone (●) and with 10 μg/ml anti-Tac antibody (○) on HuT-102 cells.

SUMMARY OF THE INVENTION

The present invention provides recombinant double chain immunotoxins comprising two components, an antigen binding component and a bacterial toxin polypeptide component. The antigen binding component is formed from two polypeptide chains (preferably immunoglobulin heavy and light chains), each chain comprising complementarity determining regions (CDRs) in

an immunoglobulin framework region. Preferred examples of such two chain antigen binding components are Fv and Fab with one chain being in peptide linkage to a bacterial toxin polypeptide component, which may be an enzyme, such as phospholipase C, or PE40.

The antigen binding component may also be chimeric or humanized. A humanized antigen binding component will have a human immunoglobulin framework region, and the CDRs may be from a different immunoglobulin than the framework region. The antigen binding site preferably binds specifically to an antigen on a cell surface, such as those on the surface of neoplastic cells or T cells, for example the Tac antigen.

Also provided by the present invention are nucleic acids encoding such immunotoxins, preferably comprising expression vectors in which a promoter is operably linked to a sequence encoding the immunotoxin; cells transformed with such expression vectors; compositions comprising such immunotoxins in a pharmaceutically acceptable carrier; and methods of treating a mammal comprising administering a therapeutically effective dose of such compositions.

DETAILED DESCRIPTION OF THE INVENTION

Antigen binding component polypeptides

The immunotoxin compositions of the present invention comprise recombinant double chain antigen binding components, preferably immunoglobulins and their fragments, and bacterial toxin components in peptide linkage to one or both chains. By the use of the term "double chain", it is understood that the immunotoxin comprises two polypeptide chains, i.e., not connected by a peptide bond, which may, however, be linked by disulfide bridges. By use of the term "recombinant" it is understood that the entire immunotoxin may be synthesized in a cell from DNA segments produced by genetic engineering. Thus, for example, recombinant immunotoxins would not comprise chemical cross-linkers such as N-succinimidyl-3-(2-pyridydithio)propionate (SPDP), thioether bonds, and the like. Complete antibodies are included as antigen binding components of such immunotoxin compositions.

Basic antibody structure

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The amino-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminus of each chain defines a constant region primarily responsible for effector function. (See, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pgs. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference.)

An "antigen binding component", as the term is used in the present invention, comprises that part of an immunoglobulin capable of specifically binding an antigen, and is formed by two immunoglobulin chains. It is preferably formed from the variable regions of each light/heavy chain pair, although antigen binding by heavy chain dimers, as well as single chains has been observed. The chains all exhibit the same general structure of relatively conserved framework regions joined by three hypervariable regions, also called CDR's (see, "Sequences of Proteins of Immunological Interest," Kabat, E. et al., U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987), which are incorporated herein by reference). The CDR's from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of two-chain forms besides complete antibodies; including various forms of modified or altered antibodies, for example: an Fv fragment, containing only the light and heavy chain variable regions; an Fab or $(Fab')_2$ fragment, containing the variable regions and parts of the

constant regions (See, generally, Hood et al., Immunology, Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323:15-16 (1986), which are incorporated herein by reference). The antigen binding components of the present invention may be of animal (especially mouse or rat), but preferably of human origin, or combinations of these as in chimeric or humanized immunoglobulins. The extensive investigation of immunoglobulin genes and their products has provided a diverse set of polypeptides exhibiting various antibody activities. For a review, see, G. Winter and C. Milstein, Nature 349:293-299 (1991), incorporated herein by reference.

"Chimeric" antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species (S. Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); incorporated herein by reference). For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as γ_1 and γ_3. A typical chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody (e.g., A.T.C.C. Accession No. CRL 9688 secretes an anti-Tac chimeric antibody), although other mammalian species may be used.

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (i.e., other than the CDR's) among different immunoglobulins in a single species, as defined by Kabat et al. (1983). As used herein, a "human framework region" is a framework region that in each existing chain comprises at least about 70 or more amino acid residues, typically 75 to 85 or more residues, identical to those in a human immunoglobulin.

As used herein, the term "humanized" or "human-like" immunoglobulin refers to an immunoglobulin comprising a human framework region and in which any constant region present is substantially identical to a human immunoglobulin constant

region (Jones et al., Nature 321:522-526, (1986), incorporated herein by reference), i.e., at least about 85-90%, preferably about 95% identical. Hence, all parts of a human-like immunoglobulin, except possibly the CDR's, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences.

An antigen specifically recognized or bound by an antigen binding component may have a wide variety of chemical structures, including peptides, nucleic acids, carbohydrates, lipids, and combinations of these (e.g., glycoproteins). Typically the antigens are membrane constituents on selected cell populations, such as neoplastic cells or T cells. More specifically, proteins on the membranes of some or all T cells that can serve as targets for the immunotoxins described here include CD2 (T11), CD3, CD4 (on helper T cells) and the IL-2 receptor (see, Leukocyte Typing III, A. J. McMichael, ed., Oxford University Press, 1987, incorporated herein by reference), and the alpha and beta chains of the T cell receptor. Proteins found predominantly on B cells that might serve as targets include CD10 (CALLA antigen), CD19 and CD20. CD45 is a possible target that occurs broadly on lymphoid cells.

Antigens found on cancer cells that might serve as targets include carcinoembryonic antigen (CEA), the erbB-2 (also called HER-2) receptor, the transferrin receptor, and the antigens recognized by 17-1A antibody (Herlyn et al., Proc. Natl. Acad. Sci. USA, 76:1438, 1979, incorporated herein by reference), L6 antibody (Hellstrom et al., Cancer Res. 46:3917-3923, (1986), incorporated herein by reference) and B6.2 antibody (Colcher et al., Proc. Natl. Acad. Sci. USA, 78:3199, (1981), incorporated herein by reference). Other protein targets on cancer cells are described in the Abstracts of the Third International Conference on Monoclonal Antibody Conjugates for Cancer (San Diego, CA, 1988).

Toxin Components

The preferred toxin components of the immunotoxin polypeptides of the present invention are bacterial polypeptide

toxins (see, The Specificity and Action of Animal, Bacterial and Plant Toxins, P. Cuatrecasas (ed.), London, Chapman and Hall (1976), incorporated herein by reference). Many useful toxins act to directly inhibit protein synthesis, e.g., by inactivating elongation factor-2 (diptheria toxin, Pseudomonas exotoxin A). These toxins must be internalized into the cytoplasm, and may have cell binding domains which may be removed to prevent unacceptable toxicity toward normal cells. Thus, for instance, Pseudomonas exotoxin (PE) may be modified to remove its binding domain (thus forming PE40).

Other useful bacterial toxins act by damaging the target cell membrane and thus do not have to be internalized, as in the case of phospholipase C (PLC), the alpha toxin of C. perfringens, which hydrolyzes the phospholipid phosphatidylcholine, a major membrane constituent (M. McFarlane and B. Knight, Biochem. J., 35:884-902 (1941), incorporated herein by reference). PLC apparently does not exhibit high-affinity, saturable binding to cells or have a specific cell binding domain. A detailed description of immunotoxins capable of damaging cell membranes can be found in the commonly assigned pending patent application U.S. serial number 07/380,172 filed July 14, 1989, which is incorporated herein by reference. Additional suitable bacterial toxins include those listed in Table 1 (all citations incorporated herein by reference), and Shigella toxin, botulinum toxin, tetanus toxin, cholera toxin, and E.coli heat-stable and heat-labile toxins.

Toxin components of the immunotoxin polypeptides are in peptide linkage, i.e., connected by a peptide bond, with the 5' or 3' end of either or both of the two chains forming the antigen binding site of these polypeptides, for example, with the heavy chain or light chain.

Hence, in nucleic acids encoding and expressing the immunotoxins of the present invention, the sequences encoding the toxin component and a chain of the antigen binding component are in the same reading frame and operably linked to the same promoter; when expressed, both components form part of the same polypeptide chain.

TABLE 1

Streptolysin O and S	Alouf, J.E. and Raynaud, M. (1973) Biochimie 55:1187-1193
Clostridium perfringens toxin	Smyth, C. (1975) J.Gen. Microbiol. 87:219-238
Clostridium perfringens alpha-toxin	Smyth, C. and Arbuthnott, J.P. (1974) J. Med. Microbiol. 7:41-65
Bacillus cereus phospholipase C	Johansen, T. et al. (1988) Gene 65:293-304
Pseudomonas aeruginosa phospholipase C	Liu, P.V. (1966) J. Infect. Dis. 116:112-116
Staphylococcus aureus α-toxin	Cassidy et al. (1974) Biochim. Biophys. Acta 332:413-423
Staphylococcus aureus β-toxin	Bernheimer et al.(1974) Ann. N.Y. Acad. Sci. 236:292-305
Staphylococcus aureus δ-toxin	Kantor et al. (1972) Arch. Biochem. Biophys. 151:142-156
Escherichia coli hemolysin	Rennie R.P., and Arbuthnott, J.P. (1974) J. Med. Microbiol. 7:179-188

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

Dimers (or higher multimers) of double chain recombinant immunotoxins may be formed, e.g., by disulfide bonds in the hinge region of the heavy chain constant domain. Such dimers can have the same relation to double chain recombinant immunotoxin monomers as $(Fab')_2$ fragments have to Fab fragments of antibodies. Although containing more than two chains, such dimers and multimers are comprised by the term "double chain recombinant immunotoxin," because their minimal active constituent units are such. Double chain recombinant immunotoxins can also be linked, genetically or chemically, to other protein domains to provide additional functions.

Nucleic Acids

The nucleic acids of the present invention include those encoding or expressing recombinant double chain immunotoxin polypeptides. These may be used to transform or transfect appropriate host cells for various purposes (e.g., production of the claimed immunotoxin polypeptides).

The recombinant immunotoxin polypeptides of the present invention may be made by synthetic polypeptide methods or more preferably by recombinant nucleic acid methods. Suitable synthetic DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts. 22:1859-1862 (1981), incorporated herein by reference. A double stranded fragment may then be obtained by annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Techniques for nucleic acid manipulation, such as subcloning nucleic acid sequences encoding polypeptides into

vectors and the isolation of substantially pure nucleic acids, are described generally in Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or Current Protocols in Molecular Biology, F. Ausubel et al., ed. Greene Publishing and Wiley-Interscience, New York (1987), which are incorporated herein by reference. The recombinant nucleic acid sequences used to produce fusion proteins of the present invention may be derived from natural or synthetic sequences.

A nucleic acid is termed "isolated" or rendered "substantially pure" when purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, and others well known in the art. See, Current Protocols in Molecular Biology (1987).

Many natural gene sequences are obtainable from various cDNA or from genomic libraries using appropriate probes. See, Genbank(TM), National Institutes of Health, incorporated herein by reference. Techniques for making and screening cDNA and genomic libraries to obtain gene sequences of interest are described, e.g., Current Protocols in Molecular Biology (1987).

It may be advantageous to employ the polymerase chain reaction (PCR) to synthesize gene sequences from genomic DNA or messenger RNA (mRNA), as necessary, using primers derived from published DNA sequences or degenerate primers derived from protein sequence. See, e.g., PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990), incorporated herein by reference.

Useful probes and primers may be selected from published sequences in accordance with published procedures. Synthetic oligonucleotides can be formulated by the triester method according to Matteucci et al., J. Am. Chem. Soc., 103:3185 (1981), incorporated herein by reference, or by other methods such as commercial automated oligonucleotide synthesizers. Nucleic acid probes used for constructing

nucleic acids encoding the polypeptide components of the immunotoxins of the present invention, for obtaining sequences encoding naturally occurring components from cDNA or genomic libraries, or other purposes will include an isolated nucleic acid attached to a label or reporter molecule. Probes may be prepared by nick translation, Klenow fill-in reaction, random hexamer priming, or other methods known in the art. For making probes, see, e.g., Current Protocols in Molecular Biology (1987).

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat (1983) and WP87/02671). For example, the human kappa immunoglobulin constant and J region genes and sequences are described in Heiter et al., Cell 22:197-207 (1980) and the nucleotide sequence of a human immunoglobulin $C_{\gamma 1}$ gene is described in Ellison et al., Nucl. Acid. Res. 10:4071 (1982), both of which are incorporated herein by reference. The variable regions or CDR's for producing the antigen binding components of the present invention will be similarly derived from hybridomas producing monoclonal antibodies capable of binding to the desired antigen (e.g., the human IL-2 receptor) and produced by well-known methods in any convenient mammalian source, including, mice, rats, rabbits, or other vertebrate capable of producing antibodies. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland; U.S.A., which is incorporated herein by reference).

In addition to the immunotoxin compositions specifically described herein, other substantially homologous modified immunotoxins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and S. Roberts et al, Nature

328:731-734 (1987), both of which are incorporated herein by reference). Such additional modifications, deletions, insertions and the like may be made to the sequences encoding the antigen binding and toxin components of the immunotoxins of the present invention, as will be readily appreciated by those skilled in the art. Especially, deletions or changes may be made in toxin-encoding sequences or in the linker connecting a chain of the antigen-binding component to the toxin, in order to increase the cytotoxicity of the fusion protein toward target cells or to decrease nonspecific cytotoxicity toward cells without antigen for the antibody. All such constructions may be made by methods of genetic engineering well known to those skilled in the art and may produce proteins that have differing properties of affinity, specificity, stability and toxicity that make them particularly suitable for various clinical or biological applications.

The sequences encoding the two chains of the double chain recombinant immunotoxin may be contained on a single vector or on separate vectors. Moreover the recombinant immunotoxin after synthesis may remain internal to the E. coli host cell until purified. In another embodiment of the invention, the sequences encoding these polypeptides may be preceded by a signal sequence that directs their secretion from the cell (Better et al., Science 240:1041-1043 (1988); Skerra & Pluckthun, Science 240:1038-1041 (1988), both ihcorporated herein by reference).

Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the sequence encoding an immunoglobulin chain forming the antigen binding component of the present invention may be fused to functional regions from other genes (e.g., different toxin components, marker genes, etc.) to produce fusion proteins having novel properties.

Vectors

Recombinant methods for polyp ptide synth sis commence with the construction of a replicable vector

containing nucleic acid that encodes the polypeptide. Vectors typically facilitate the cloning of the nucleic acid encoding the immunotoxin, i.e., to produce usable quantities of the nucleic acid. If a vector is an expression vector, it also directs the expression of the immunotoxin. One or both of these functions are performed by the vector-host system. The vectors will contain different components depending upon the function they are to perform as well as the host cell that is selected.

Commonly, DNA expression vectors incorporating coding regions for a polypeptide will most preferably be suitable for replication in a unicellular host, such as bacteria or possibly a yeast, but may also be intended for introduction to, and possibly introduction into the genome of, a cultured mammalian or plant or other eukaryotic cell lines. Such vectors will typically include a replication system recognized by the host, including an origin of replication or autonomously replicating sequence (ARS), the intended DNA fragment encoding the desired polypeptide, and transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment. The transcriptional regulatory sequences may include a promoter, heterologous enhancer and necessary processing information sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences and mRNA stabilizing sequences, all of which are recognized by the host. The polypeptide encoding segment may be preceded by such translational initiation sequences such as ribosome-binding sites. Such expression vectors may also include secretion signals from secreted polypeptides of the same or related species, which allow the protein to cross cell membranes, and thus attain its functional topology.

The selection of an appropriate promoter will depend upon the host, but for bacterial hosts, bacterial promoters such as the phoA, trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known and commonly used (See, Molecular Cloning: A Laboratory Manual (1989)). Useful yeast promoters include the promot r r gions for metallothionein; 3-phosphoglycerate kinase or other glycolytic

enzymes such as enolase, glyceraldehyde-3-phosphate dehydrogenase; enzymes responsible for maltose and galactose utilization; and others. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EP 73,657A, incorporated herein by reference. Appropriate non-native mammalian promoters might include the early and late promoters of SV40 or promoters derived from mouse mammary tumor virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma virus, among others. Examples of workable combinations of cell lines and expression vectors are described in Molecular Cloning: A Laboratory Manual (2nd ed.) (1989); see also, Metzger et al., Nature 334:31 (1989), incorporated herein by reference.

Selectable markers

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selectable markers, genes encoding a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures the growth of only those host cells which express the marker activity. Typical selectable marker genes encode proteins that (a) confer resistance to antibiotics or other toxic substances, e.g., ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

Host cells

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. Other microbes, such as yeast (e.g., Saccharomyces), may also be used for expression.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). A number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc, but preferably transformed B-cells or hybridomas.

Transformation of host cells

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent or capable of being mobilized to another cell by functions supplied in trans); microinjection; and other methods are commonly utilized (See, generally, Molecular Cloning: A Laboratory Manual (2nd ed.) (1989), or Current Protocols in Molecular Biology (1987)).

Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the double chain recombinant immunotoxin may follow.

Methods of producing and purifying immunotoxins suitable for use in the present invention are well known to

those skilled in the art and can be found described in such publications as Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, NY, Cold Spring Harbor Press, 1989, incorporated herein for all purposes by reference. In general, methods for protein purification, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like are described in R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure polypeptides of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or other purposes (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

Methods of Use

The immunotoxin compositions of the present invention may also be used in combination with other antibodies, or immunotoxins, particularly human monoclonal antibodies reactive with other markers on cells responsible for the disease. For example, suitable T-cell markers can include those grouped into the so-called "Clusters of Differentiation," as named by the First International Leukocyte Differentiation Workshop, Leukocyte Typing, Bernard et al., Eds., Springer-Verlag, N.Y. (1984), which is incorporated herein by reference.

The immunotoxin compositions can also be used as separately administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. Typically, the agents will include cyclosporin A or a purine analog (e.g., methotrexate, 6-mercaptopurine, or the like), but numerous additional agents (e.g., cyclophosphamide, prednisone, etc.) well-known to those skilled in the art may also be utilized.

As the antigen binding component of the immunotoxins of the present invention, intact immunoglobulins or their binding fragments, such as Fv or Fab, are preferably used. When entire antibodies are employed, they will typically be of

the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The compositions of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the immunotoxin or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as human albumin, pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of immunotoxin in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and 5 mg of immunotoxin. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 15 mg of immunotoxin. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The immunotoxin compositions of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization

and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees loss of activity (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions of the present invention or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the infection and the general state of the patient's own immune system, as well as on the particular immunotoxin employed in treatment and the therapeutic purpose, but typically range from about 0.1 to about 20 mg of immunotoxin per dose, with dosages of from 1 to 5 mg per patient being more commonly used. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases it is possible and may be felt desirable by the treating physician to administer substantial excesses of these immunotoxins.

In prophylactic applications, compositions containing the present immunotoxins or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 5 mg per dose, especially 0.5 to 2.5 mg per patient.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmac utical formulations should provide a quantity of the

immunotoxin(s) of this invention sufficient to effectively treat the patient.

Anti-Tac Specificity

An exemplary embodiment of the present invention is the use of immunotoxins directed against the IL-2 receptor. The anti-Tac antibody recognizes the p55 chain of the human IL-2 receptor (T. Uchiyama et al., J. Immunol. 126:1393-1397 (1981), incorporated herein by reference).

The IL-2 receptor specific immunotoxins exemplified in the present invention will typically find use individually in treating a T-cell mediated disease state. Generally, where the cell linked to a disease has been identified as IL-2 receptor bearing, such immunotoxins may be used. For example, typical disease states suitable for treatment include graft versus host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lungs, kidneys, liver, etc. Other diseases include autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis; and malignancies such as adult T cell leukemia, Hodgkin's disease, and other lymphomas.

EXAMPLE 1. ANTI-TAC(Fab)-PLC

An important class of toxins are the cytolytic toxins produced by many bacteria (J. Alouf, in: P. Cuatrecasas (ed.), The Specificity and Action of Animal, Bacterial and Plant Toxins, pp. 221-270. London, England: Chapman and Hall (1976)), which act by damaging the target cell membrane and thus do not have to be internalized. We utilized phospholipase C (PLC), the alpha toxin of C. perfringens. PLC is a highly potent toxin that causes cell damage by hydrolyzing the phospholipid phospatidylcholine, a major membrane constituent (M. MacFarlane and B. Knight, Biochem. J. 35:884-902 (1941), incorporated herein by reference). The PLC gene has been previously cloned and expressed it in E. coli (J. Tso and C. Siebel, Infection and Immunity 57:468-476 (1989), incorporated herein by reference). PLC apparently does not exhibit high-affinity,

saturable binding to cells or have a specific cell binding domain.

PLC has been linked to the Fab fragment of the anti-Tac antibody, which recognizes the p55 chain of the human IL-2 receptor (T. Uchiyama et al. (1981)), to produce an anti-Tac(Fab)-PLC immunotoxin. Anti-Tac(Fab)-PLC specifically binds to and inhibits protein synthesis in human T cells expressing the IL-2 receptor.

A. Construction of anti-Tac(Fab)-PLC

Antibody Fab fragments can be expressed and secreted from E. coli (Better et al. (1988)). Hence, we attached the complete PLC gene with its own promoter and signal sequence (J. Tso and C. Siebel (1989)) to the V_H-C_H1 domains of the cloned anti-Tac heavy chain gene (C. Queen et al., Proc. Natl. Acad. Sci. USA 86:10029-10033 (1989), incorporated herein by reference), followed by a termination codon (Fig. 1). For ease of DNA manipulation and to provide a spacer between the PLC and antibody proteins, a 27 bp linker encoding 9 amino acids was inserted between PLC and V_H. The C_H1 domain was followed on the same cistron by the mature anti-Tac light chain gene, attached to the Shine and Dalgarno (ribosome binding) sequence, initiation codon and signal coding sequence of E. coli heat-stable enterotoxin II (R. Picken et al., Infection and Immunity 42:269-275 (1983), incorporated herein by reference). PLC was fused to the amino rather than the carboxy end of the antibody because preliminary experiments had shown that the amino terminal end of PLC itself must be free if the enzyme is to retain activity.

This transcriptional unit was inserted into the EcoRI site of pBR322, as described below.

1. Plasmid construction

The plasmid PLCTAC, which encodes anti-Tac(Fab)-PLC, was constructed from five consecutive DNA fragments (Fig. 1). First, in vitro mutagenesis in M13 (M. Zoller and M. Smith, Nucl. Acids Res. 10:6487-6500 (1982), incorporated herein by reference) was used to attach an ApaI restriction site after

the terminals Lys codon of the PLC gene (J. Tso and C. Siebel (1989)), and a fragment extending from an EcoRI site upstream of the PLC promoter down to the new ApaI site was purified. In vitro mutagenesis in M13 was also used to modify the cDNA of the anti-Tac heavy chain (C. Queen et al. (1989)): a BstEII site and codons for the amino acids Lys and Ala were inserted just 5' to the Gln codon that starts the mature coding sequence of the heavy chain, and a stop codon and XbaI site were inserted immediately after the C_H1 coding region. The PLC EcoRI - ApaI fragment was combined with the anti-Tac heavy chain BstEII - XbaI fragment by means of an ApaI - BstEII linker fragment containing the sequence GGGCCCGACAGCACCGGTGACC, synthesized on an Applied Biosystems Model 380B Synthesizer. Next, a short XbaI - MluI fragment containing the Shine and Dalgarno ribosome binding sequence and most of the signal coding sequence of E. coli heat-stable enterotoxin II (ST II) (R. Picken et al. (1983)) was produced by oligonucleotide synthesis. In vitro mutagenesis was used to insert an MluI site and the remaining part of the ST II signal sequence 5' to the mature light chain coding sequence of anti-Tac (C. Queen et al. (1989)), and to insert an EcoRI site after the termination codon of the light chain. The ST II XbaI - MluI fragment was combined with the light chain MluI - EcoRI fragment in the plasmid pUC19. The combined EcoRI - XbaI fragment containing PLC and the heavy chain was then ligated with the combined XbaI - EcoRI fragment containing the ST II sequences and the light chain, and was inserted in the EcoRI site of pBR322 to produce PLCTAC.

B. Purification of anti-Tac(Fab)-PLC

Osmotic shock extract was prepared in H_2O as described (J. Tso and C. Siebel (1989)) from E. coli cells containing the plasmid PLCTAC, in order to isolate proteins located in the periplasmic space. The extract was adjusted to 50 mM in Tris, pH 8.0 and applied to a column of Affi-Gel 10 coupled with rabbit anti-(mouse kappa chain) antibody (Cappel). The column was washed with 0.1 M glycine, pH 3.0. Anti-

Tac(Fab)-PLC was eluted with 3.5 M $MgCl_2$ and dialyzed against 20 mM Tris, pH 8.0.

C. Enzymatic assays

Phospholipase C enzymatic activity was determined as described (J. Tso and C. Siebel (1989)), using an ethanoic dispersion of phosphatidylcholine substrate. One unit of activity equals one micromole of product formed per minute at 37°C. Hemolytic activity of PLC was determined using sheep erythrocytes as described (J. Tso and C. Siebel (1989)). One 50% hemolysis unit is the amount of enzyme that causes 50% hemolysis of the erythrocytes under the described conditions, measured spectrophotometrically at 550 nm.

D. Binding assay

The affinity of anti-Tac(Fab)-PLC for the IL-2 receptor was determined as described (C. Queen et al. (1989)). Briefly, increasing amounts of competitor (anti-Tac antibody or anti-Tac(Fab)-PLC) were added to 1.5 ng of radioiodinated tracer anti-Tac antibody (2 uCi/ug) and incubated with 4 x 10^5 Hut-102 cells in 0.2 ml binding buffer (RPMI 1040 medium with 10% fetal calf serum, murine Ig at 10 ug/ml, 0.1% sodium azide) for 2 hours at 0°C. Cells were washed and pelleted, their radioactivities measured, and the concentrations of bound and free tracer antibody calculated. The affinity constant of anti-Tac(Fab)-PLC was then calculated (C. Queen et al. (1989)).

E. Cytotoxicity assay

10^5 cells were plated in 200 ul of RPMI medium with 5% calf serum in each well of a 96-well plate. Various amounts of PLC or anti-Tac(Fab)-PLC were added to each well in 20 ul RPMI medium, and the cells were incubated for 16 hours at 37°C. 2 uCi ^{3}H-leucine was added to each well and the cells incubated an additional 4 hours. The radioactivity in a trichloroacetic acid precipitate of each cell pellet was determined.

F. Properties of anti-Tac(Fab)-PLC

Cells containing the plasmid PLCTAC were grown and protein extracted from the periplasmic space by osmotic shock as previously described (J. Tso and C. Siebel (1989)). Western blot analysis using goat anti-(mouse Ig) antibody identified a 90 kD protein under non-reducing conditions, corresponding to the predicted molecular weight of PLC linked to a Fab fragment. A 65 kD band was also identified, most likely representing free PLC-heavy chain, since a reducing gel showed this 65 kD band and a 25 kD light chain band. Evidently, either the light chain was not expressed in equimolar amounts with the PLC-heavy chain, or protein assembly was inefficient and free light chain was degraded. To avoid copurifying the free PLC-heavy chain, the anti-Tac(Fab)-PLC protein was purified from the osmotic shock extract using a rabbit anti-(mouse kappa chain) affinity column, as described above. The resulting protein was about 90% pure as judged by a coomassie stained gel (Fig. 2). The enzymatic and binding activities of the purified anti-Tac(Fab)-PLC protein were assessed. The PLC in the anti-Tac(Fab)-PLC conjugate had about 4-fold less enzymatic activity on phosphatidylcholine and about 2-fold less hemolytic activity on sheep erythrocytes than free PLC (Table 2). Binding to IL-2 receptor was measured using Hut-102 cells, a human T-cell lymphoma line expressing the IL-2 receptor p55 chain at a high level (Uchiyama et al. (1981)). Increasing amounts of competitor (anti-Tac antibody or anti-tac(Fab)-PLC) were added to radio-labeled tracer anti-Tac and incubated with the Hut-102 cells (Fig. 3). The affinity constant of anti-Tac(Fab)-PLC was calculated as $8 \times 10^7\ M^{-1}$ versus $3 \times 10^9\ M^{-1}$ for anti-Tac itself, a 37-fold loss in affinity.

TABLE 2

Specific activity of anti-Tac(Fab)-PLC[a]

Toxin	Hemolytic activity (HU_{50}/nmole)	Enzymatic activity (U/nmole)
PLC	9174	57
Anti-Tac(Fab)-PLC	4166	13

[a]Assays and units are as described (J. Tso and C. Siebel (1989)).

The immunotoxin was tested for cytotoxicity on HuT-102 cells (Fig. 4). Anti-Tac(Fab)-PLC inhibited protein synthesis in a dose-dependent manner with 50% inhibitory concentration (IC_{50}) of 0.02 nM (1.8 ng/ml). In contrast, the IC_{50} of PLC alone was 25 nM, so linking PLC to the anti-Tac targeting antibody increased its toxicity on HuT-102 cells by more than 1000-fold. Competition with 10 ug/ml anti-Tac abolished the increased cytotoxicity of anti-Tac(Fab)-PLC, demonstrating specificity of targeting (Fig. 4). Moreover, the human T-cell leukemia line CEM, which does not express the Tac antigen p55, could not be inhibited by concentrations of anti-Tac(Fab)-PLC as high as 10 nM. It is important to note that the inhibition of protein synthesis measured by the cytotoxicity assay is an indirect result of cell membrane damage by PLC, rather than the direct enzymatic effect of toxins such as ricin. Hence, it is possible that this standard assay understates the effect of membrane-acting immunotoxins relative to conventional ones.

C. perfringens

PLC, a membrane-acting toxin, has previously been linked to polyclonal antibodies against sheep red blood cells using chemical methods (F. Moolten et al., Immunol. Rev. 62:47-73 (1982), incorporated herein by reference). However, concentrations of 1 mg/ml were required to lyse the red blood cells, and the authors concluded that this chemically

conjugated immunotoxin lacked potency, in contrast to the recombinant immunotoxin anti-Tac(Fab)-PLC disclosed herein.

Care will be required when using immunotoxins based on PLC in vivo, because of the hemolytic properties of PLC. However, the selective increase in cytotoxicity obtained by genetically linking PLC to an antibody may make it possible to use such an immunotoxin at doses that destroy the targeted cells while leaving red blood cells and other normal cells undamaged.

EXAMPLE 2. ANTI-TAC(Fab)-PE40

A. Construction of anti-Tac(Fab)-PE40

Anti-Tac(Fab)-PE40 is a recombinant, two-chain immunotoxin in which PE40 is genetically linked to the Fab fragment of anti-Tac (Fig. 5). The plasmid pTACPE40, which encodes anti-Tac(Fab)-PE40, was constructed from five consecutive DNA fragments (Fig. 6). First, in vitro mutagenesis in M13 was used to create a BstEII site by mutating the third nucleotides encoding amino acids Pro (-5) and Thr (-3) of the alkaline phosphatase (phoA) signal sequence, and a HindIII site located 5' upstream of the phoA promoter was converted into an EcoRI site. The resulting EcoRI-BstEII fragment contains the phoA promoter and signal codons. In vitro mutagenesis in M13 was also used to modify the cDNA of the anti-Tac heavy chain: a BstEII site and codons for the amino acids Lys and Ala were inserted just 5' to the Gln codon that starts the mature coding sequence of the heavy chain, and a step codon and XbaI site were inserted immediately after the C_H1 coding region. The phoA EcoRI-BstEII fragment was combined with the anti-Tac heavy chain BstEII-XbaI fragment in the plasmid pUC19.

Next, a short XbaI-MluI fragment containing the Shine and Dalgarno ribosome binding sequence and most of the signal coding sequence of E. coli heat-stable enterotoxin II was produced by oligonucleotide synthesis. Then, in vitro mutagenesis was used to insert an MluI site and the remaining part of the STII signal coding sequence 5' to the mature light chain coding sequence of anti-Tac, and to insert the sequence

GTCGACGCGGGCCCATGG (containing an NcoI site) immediately after the last codon (Cys) of the light chain. In vitro mutagenesis was also used to insert a NcoI site just 5' to the Gly codon that starts domain II of the Pseudomonas exotoxin (PE40) gene. The fragment extending from the NcoI site to an EcoRI site located at the 3' end of the PE gene was isolated. The above-mentioned three fragments -- the STII XbaI-MluI fragment, the light chain MluI-NcoI fragment, and the PE40 NcoI-EcoRI fragment -- were combined in the plasmid pUC19.

The combined EcoRI-XbaI fragment containing the PhoA promoter and signal sequence and the heavy chain was then ligated with the combined XbaI-EcoRI fragment containing the STII sequence, the light chain and the PE40 sequence, and finally inserted in the EcoRI site of pBR322 to produce the plasmid pTACPE40.

B. Purification of anti-Tac(Fab)-PE40

Osmotic shock extract was prepared in H_2O as described previously (J. Tso & C. Siebel (1989)) from E. coli cells containing the plasmid pTACPE40, in order to isolate proteins located in the periplasmic space. The extract was adjusted to 50 mM in Tris-HCl, pH 8.0 and applied to a column of Sepharose 4B coupled with rat anti-(mouse kappa light chain) antibody (Zymed). Anti-Tac(Fab)-PE40 was eluted with 3.5 M MgCl2 and dialyzed against 20 mM Tris-HCl, pH 8.0. The protein was estimated by SDS-polyacrylamide gel analysis to be 20% pure.

Alternatively, for greater purity the anti-Tac(Fab)-PE40 is purified from the extract by chromatography on S-Sepharose in 25 mM MES, pH 6.5, eluting with 300 mM NaCl, and then by chromatography on Q-Sepharose in 20 mM bis-Tris, pH 6.5, eluting with a 0-400 mM NaCl gradient.

C. Cytotoxicity assay

10^5 HuT-102 cells were plated in 200 µl of RPMl medium with 5% calf serum in each well of a 96-well plate. Various amounts of anti-Tac(Fab)-PE40 with or without 10 µg/ml of anti-Tac were added to each well in 20 µl RPMI medium, and

the cells were incubated for 16 hr at 37°C. 2 μCi of ^{3}H-leucine was added to each well. The cells were further incubated for 4 hr at 37°C and harvested onto glass fiber filters by means of a PHD cell harvester (Cambridge Technology), washed with water, dried and counted. The results are expressed as a percentage of the ^{3}H-leucine incorporation in control cultures, which have not been incubated with immunotoxin (Fig. 7). Increasing concentrations of anti-Tac(Fab)-PE40 inhibit protein synthesis in the cells (IC_{50}= 0.2 ng/ml). Competitor anti-Tac antibody prevents this inhibition, showing the specificity of anti-Tac(Fab)-PE40.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Queen, Cary L.

(ii) TITLE OF INVENTION: Recombinant Double Chain Immunotoxins

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/666,287
(B) FILING DATE: 08-MAR-1991
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Smith, William M.
(B) REGISTRATION NUMBER: 30,223
(C) REFERENCE/DOCKET NUMBER: 11823-29

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-326-2400
(B) TELEFAX: 415-326-2422

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..33

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(B) LOCATION: 1..24

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(ii) MOLECULE TYPE: protein

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1 5

WHAT IS CLAIMED IS:

1. A substantially pure recombinant immunotoxin comprising an antigen binding component and a bacterial toxin polypeptide component, said antigen binding site formed by two polypeptide chains, each comprising CDRs in an immunoglobulin framework region, wherein said toxin component is in peptide linkage to one of the chains.

2. An immunotoxin composition of Claim 1 wherein said antigen binding component is an Fv or an Fab.

3. An immunotoxin composition of Claim 1 wherein said antigen binding component is a chimeric antigen binding component.

4. An immunotoxin composition of Claim 1 wherein the framework region is a human immunoglobulin framework region.

5. An immunotoxin composition polypeptide of Claim 1 wherein the CDRs are from a different immunoglobulin than the framework region.

6. An immunotoxin composition of Claim 1 wherein said antigen binding component binds specifically to an antigen on the surface of a cell.

7. An immunotoxin composition of Claim 7 wherein said cell is a neoplastic or T cell.

8. An immunotoxin composition of Claim 7 wherein said antigen binding component comprises the variable domains of anti-Tac.

9. An immunotoxin composition of Claim 1 wherein said toxin is an enzyme.

10. An immunotoxin composition of Claim 1 wherein said toxin is phospholipase C or PE40.

11. An immunotoxin composition consisting essentially of anti-Tac(Fv) and a toxin, wherein said anti-Tac(Fv) comprises two chains, one chain of which is in peptide linkage to said toxin.

12. An immunotoxin composition of Claim 12 wherein said toxin is PE40.

13. An immunotoxin composition consisting essentially of anti-Tac(Fab) and a toxin, wherein said anti-Tac(Fab) comprises two chains, one chain of which is in peptide linkage to said toxin.

14. An immunotoxin composition of Claim 14 wherein said toxin is PE40.

15. A composition capable of killing cells expressing a human IL-2 receptor consisting essentially of an immunotoxin of Claims 12 or 14 or mixtures thereof.

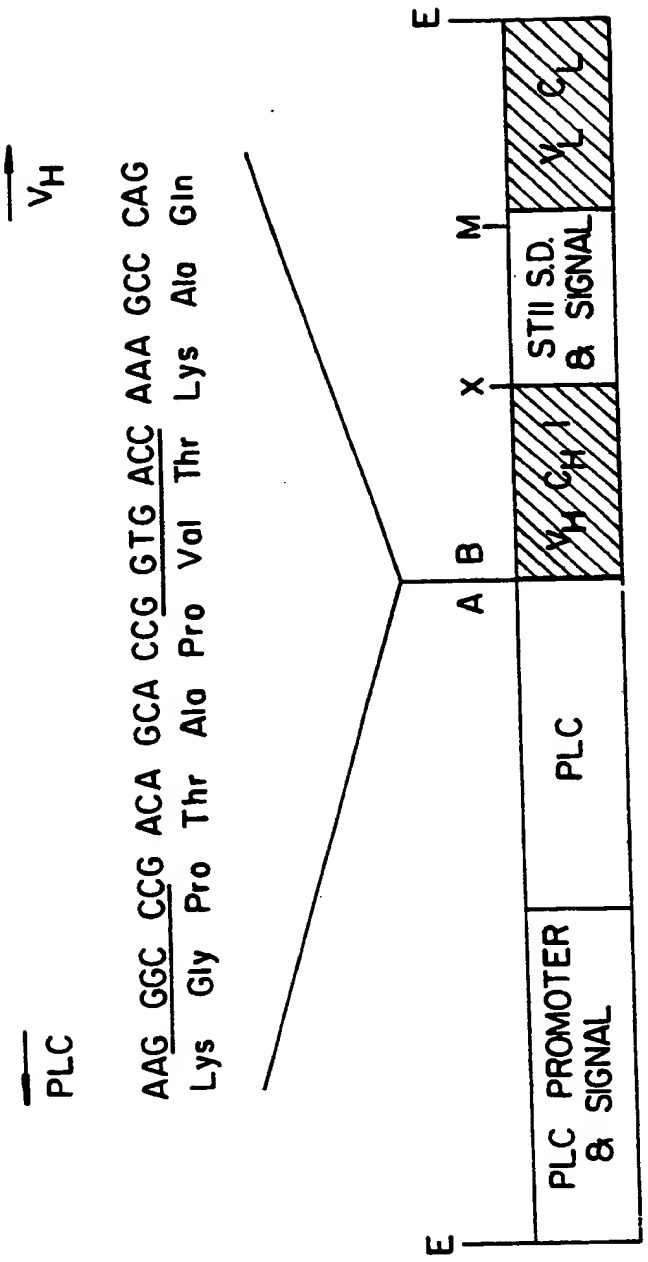
16. An isolated nucleic acid encoding an immunotoxin polypeptide of Claims 1, 12, or 14.

17. An isolated nucleic acid comprising an expression vector having a promoter operably linked to a sequence encoding an immunotoxin of Claims 1, 12, or 14.

18. A cell transformed or transfected with a nucleic acid comprising an expression vector having a promoter operably linked to a recombinant DNA sequence encoding an immunotoxin comprising an antigen binding component and a bacterial toxin polypeptide component, said antigen binding component formed by two polypeptide chains, each chain comprising complementarity determining regions (CDRs) in an immunoglobulin framework region, and said toxin is in peptide linkage to one of the chains.

19. A composition comprising a recombinant immunotoxin of Claim 1 in a pharmaceutically acceptable carrier.

20. A method of treating a mammal comprising administering a therapeutically effective dose of a composition of Claim 19.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E
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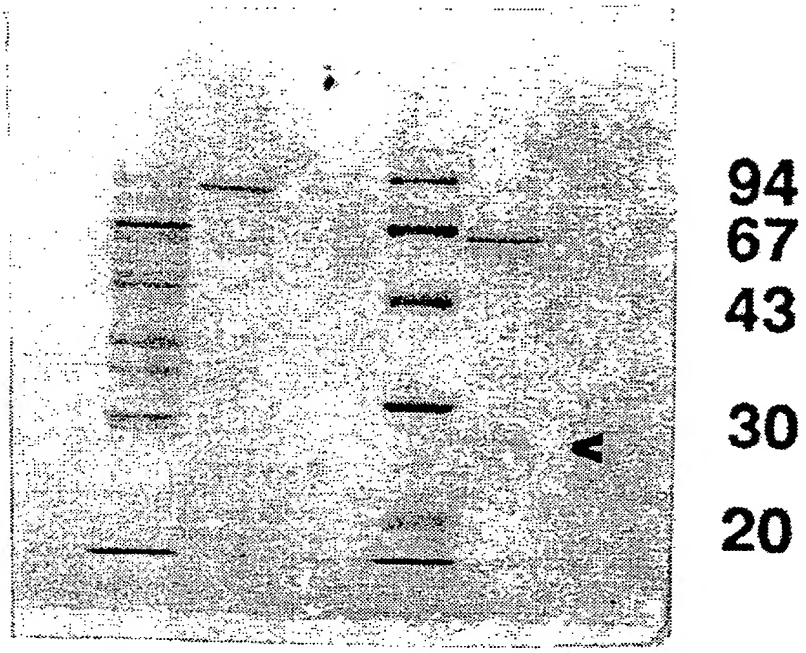
V_L C_L

FIG. 1.



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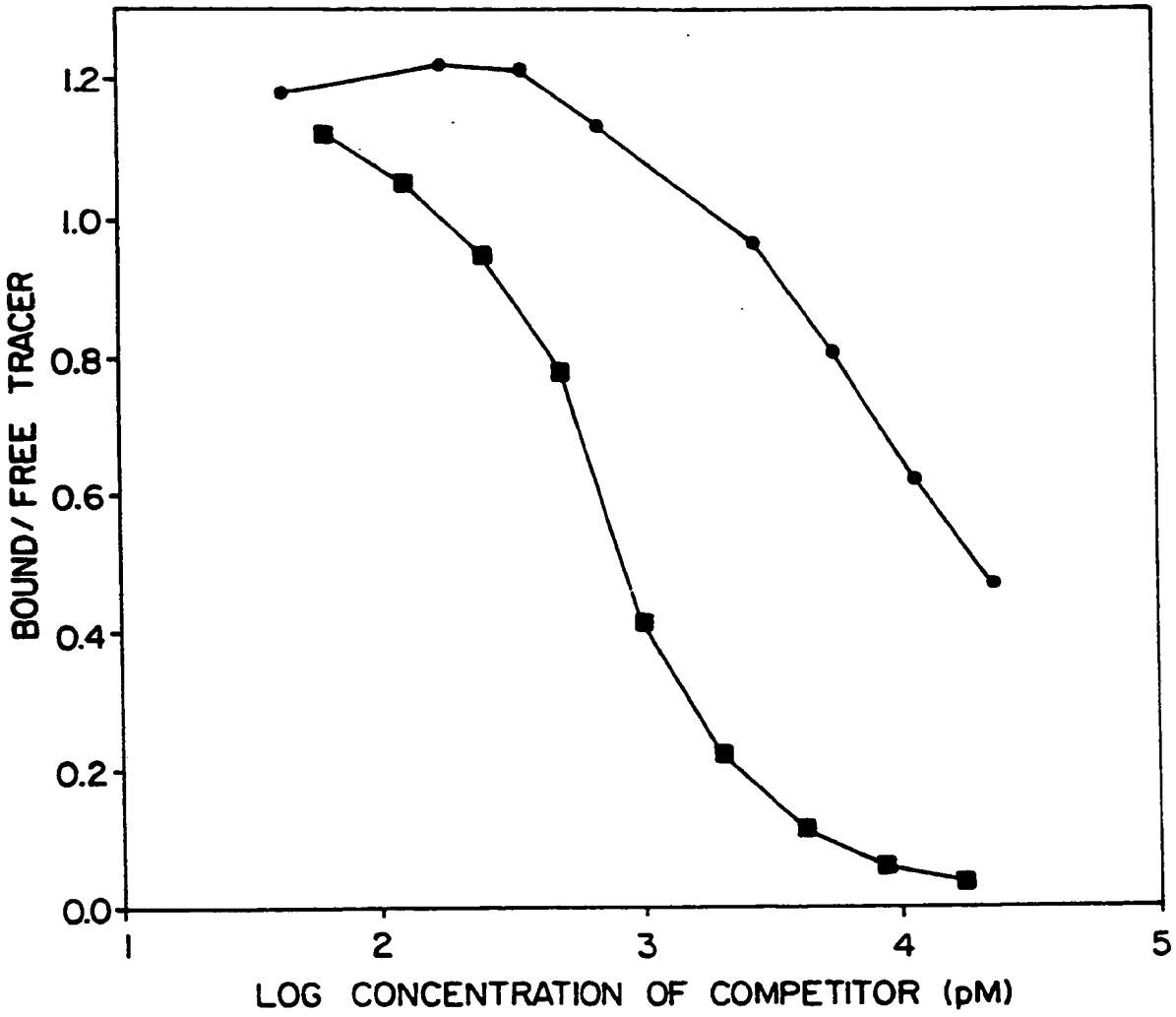
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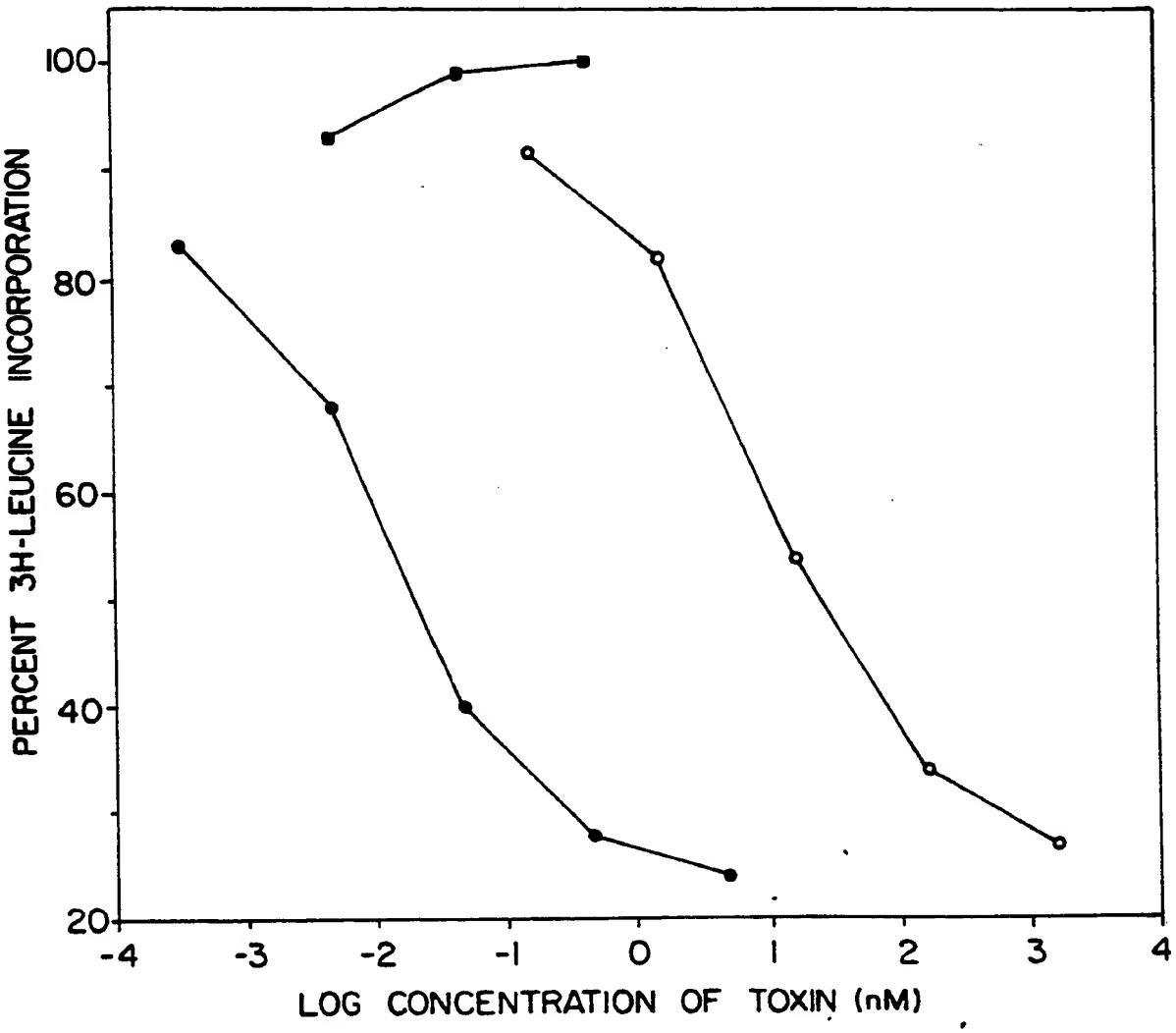
LOG CONCENTRATION OF COMPETITOR (pM)

FIG. 3.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40
20
PERCENT 3H-LEUCINE INCORPORATION
-4
-3
-2
-1
0
1
2
3
4
LOG CONCENTRATION OF TOXIN (nM)

FIG. 4.

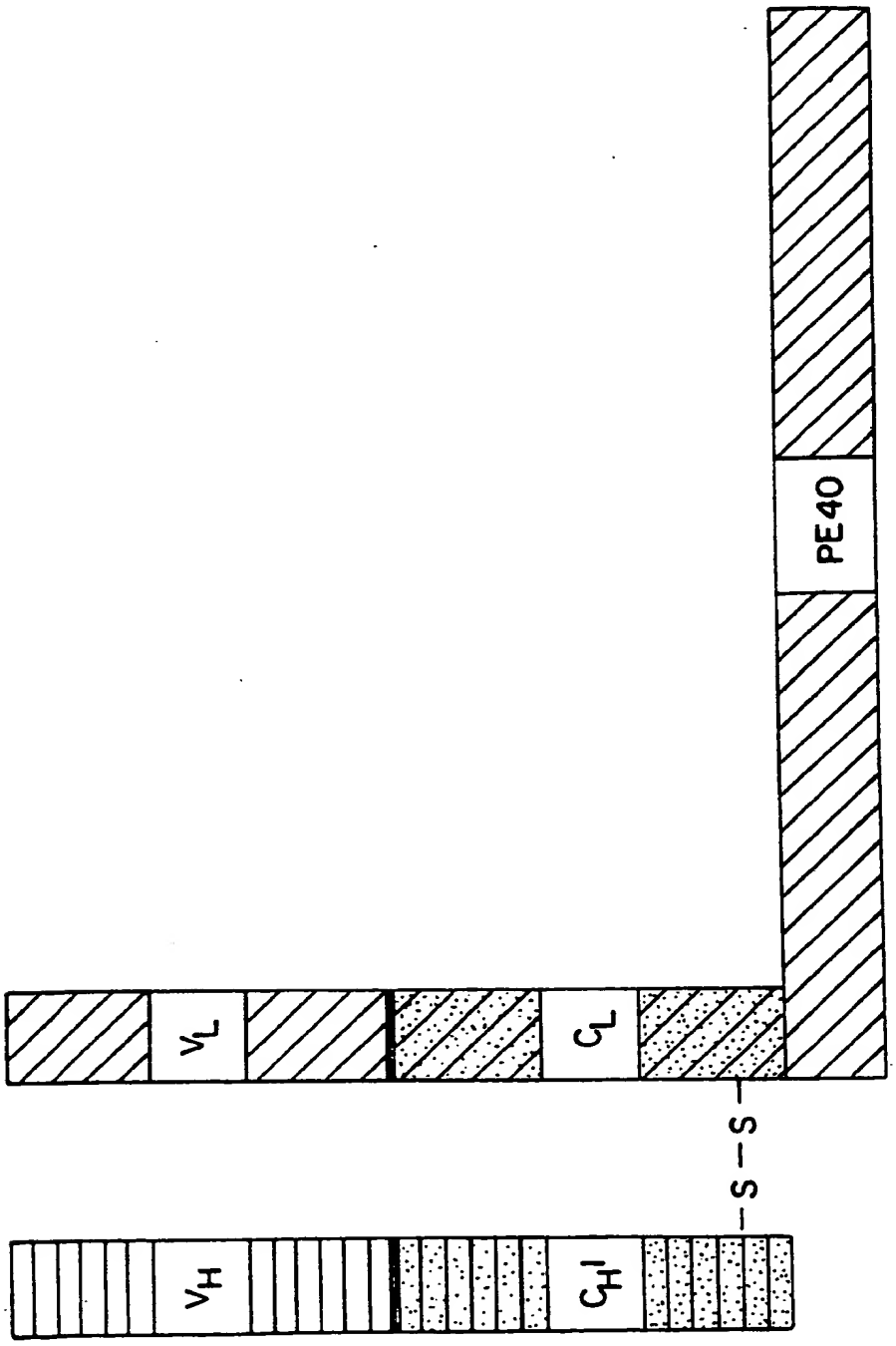

FIG. 5.

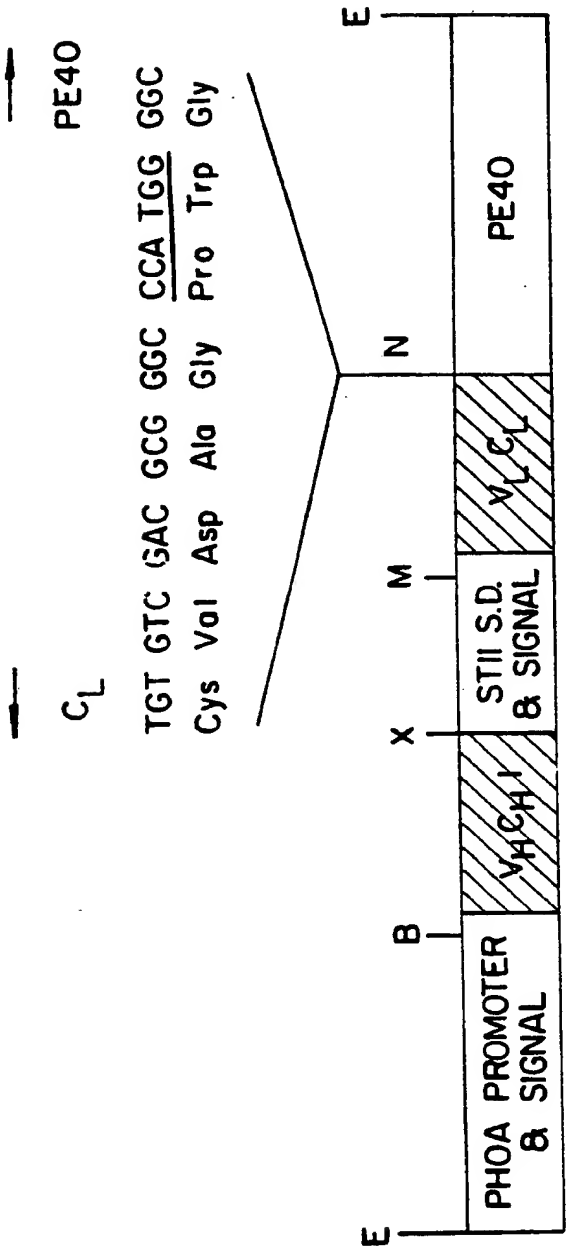
E
PE40
N
M
X
B
E
PHOA PROMOTER & SIGNAL
STII S.D. & SIGNAL
$V_H C_H I$
$V_L C_L$
PE40
C_L
TGT GTC GAC GCG GGC GGC CCA TGG GGC
Cys Val Asp Ala Gly Gly Pro Trp Gly

FIG. 6.

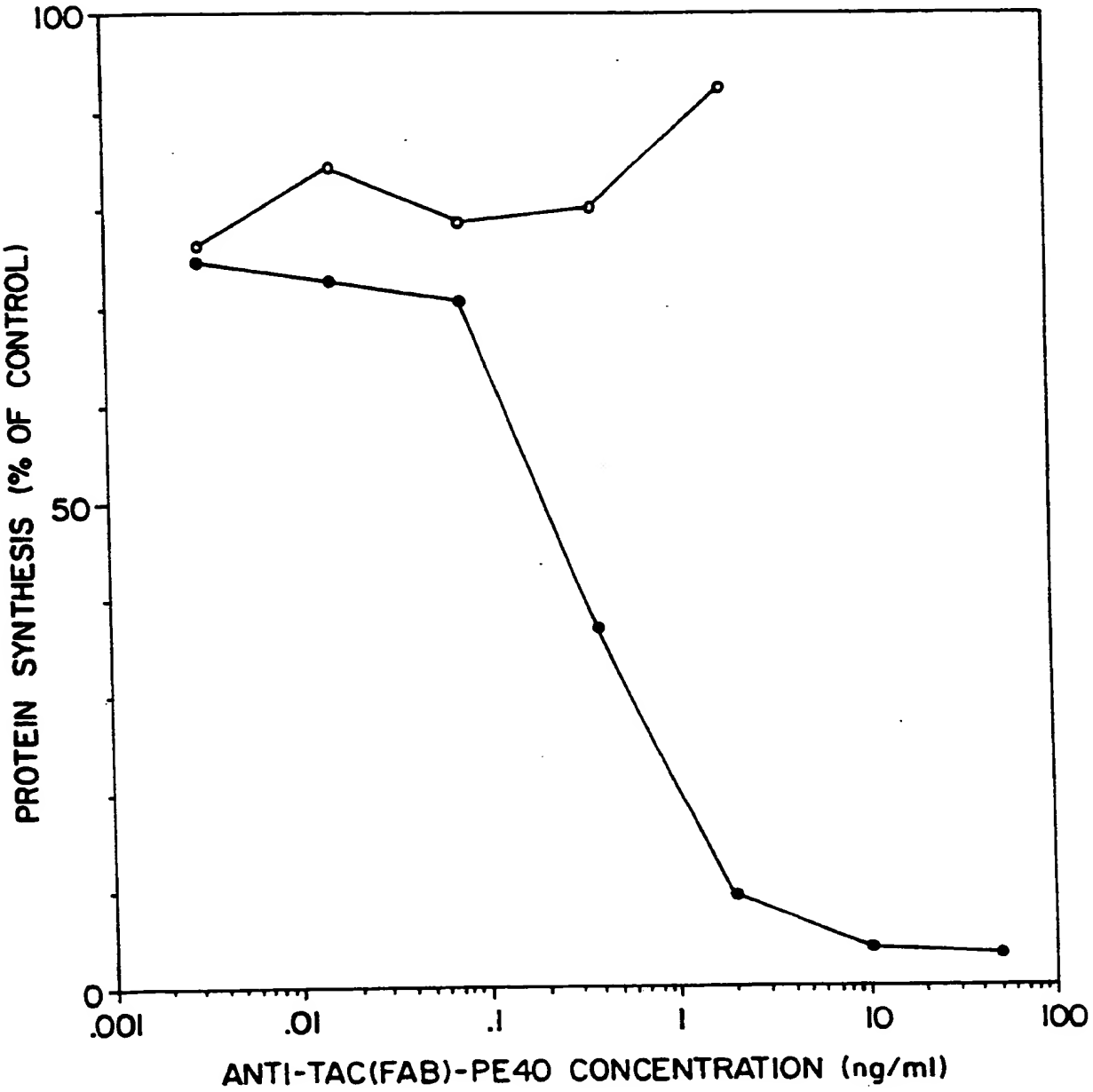
100
50
0
PROTEIN SYNTHESIS (% OF CONTROL)
.001
.01
.1
1
10
100
ANTI-TAC(FAB)-PE40 CONCENTRATION (ng/ml)

FIG. 7.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01784

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K39/00
US CL : 530/388.8, 391.9, 391.7, 867; 424/85.8, 85.91; 514/12

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/388.8, 391.9, 391.7, 867; 424/85.8, 85.91; 514/12

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

CAS ONLINE, MEDLINE, APS, DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	Cancer Research, Volume 51, issued 15 January 1991, Chovnick et al, "A recombinant, membrane-acting immunotoxin, pages 465-467, see entire document.	1-20
X	Proc. Natl. Acad. Sci. USA, Volume 87, issued November 1990, Kreitman et al, "The recombinant immunotoxin anti-Tac(Fv)-Pseudomonas exotoxin 40 is cytotoxic toward peripheral blood malignant cells from patients with adult T-cell leukemia", pages 8291-8295, see entire document.	1-20
X	Nature, Volume 339, issued 01 June 1989, Chaudhary et al, "A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin", pages 394-397, see entire document.	1-20
X	US, 90-463-111 (I. Pastan) 11 Jan 1990, see entire document.	1-20
X	US, 89-454162 (I. Pastan) 21 Dec 1989, see entire document.	1-20

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
21 MAY 1992	26 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	GREGORY P. EINHORN

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET		
A	Proc. Natl. Acad. Sci. USA, issued 01 Feb 1990, Chaudhary et al, "A rapid method of cloning functional variable-region antibody genes in Escherichia coli as single-chain immunotoxins, Pages 1066-1070, see entire document.	1-20

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.